MOLECULAR AND CELLULAR BIOLOGY, Apr. 2007, p. 2910–2918 Vol. 27, No. 8
0270-7306/07/$08.00+0 doi:10.1128/MCB.02256-06

Interferon Regulatory Factor 7 Is Activated by a Viral Oncoprotein through RIP-Dependent Ubiquitination▽

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Received 1 December 2006/Returned for modification 17 January 2007/Accepted 2 February 2007

As a key mediator of type I interferon (IFN) (IFN-α/β) responses, IFN regulatory factor 7 (IRF7) is essential to host immune defenses. Activation of IRF7 generally requires virus-induced C-terminal phosphorylation, which leads to its nuclear accumulation and activation of target genes. Here we use the Epstein-Barr virus (EBV) oncoprotein LMP1, which activates IRF7, to identify factors involved in IRF7 activation. We demonstrate for the first time that RIP activates IRF7 and that RIP and IRF7 interact under physiological conditions in EBV-positive Burkitt's lymphoma cells. We provide evidence that both RIP and IRF7 are ubiquitinated in these cells and that IRF7 preferentially interacts with ubiquitinated RIP. RIP is required for full activation of IRF7 by LMP1, with LMP1 stimulating the ubiquitination of RIP and its interaction with IRF7. Moreover, LMP1 stimulates RIP-dependent K63-linked ubiquitination of IRF7, which regulates protein function rather than proteasomal degradation of proteins. We suggest that RIP may serve as a general activator of IRF7, responding to and transmitting the signals from various stimuli, and that ubiquitination may be a general mechanism for enhancing the activity of IRF7.

Type I interferons (IFN), IFN-α/β, are critically important in the defense against viral and bacterial infections and regulate both innate and adaptive immune responses as well as affecting cell growth, differentiation, and survival (3, 29). Key regulators of IFN-α/β expression are IFN regulatory factor 7 (IRF7) and IRF3. IRF3 is constitutively expressed in most cell types and is responsible for the induction of the early phase of IFN-α/β production after infection. IRF7 is expressed constitutively at low levels in lymphoid cells and can be induced to high levels in other cell types by IFN-α/β, lipopolysaccharide, tumor necrosis factor alpha (TNF-α), tetradecanoyl phorbol acetate, virus infection, and the Epstein-Barr virus (EBV) oncoprotein LMP1 (1, 23, 24, 44). The induced IRF7 primes the cells for a second, more diverse wave of IFN-α/β production (26). IRF7 controls all type I IFN-dependent immune responses (11), which emphasizes its centrality in host immune defenses and highlights the need to understand the mechanisms regulating its activity.

Viral infection, Toll-like receptor (TLR) signaling, DNA-damaging agents, and EBV LMP1 activate IRF7, marked by its phosphorylation and accumulation in the nucleus (1, 8, 10, 16, 18, 32, 45). The IκB kinase (IKK)-related kinases IKKε and TBK1 phosphorylate C-terminal serine residues in IRF7 in response to viral infection (34). In addition, IRAK1 phosphorylates IRF7 in vitro and contributes to its activation by TLR7/9 (37).

EBV, a gammaherpesvirus, is a human tumor virus. In addition to its lytic cycle in epithelial cells, a hallmark of EBV infection is latent infection of B lymphocytes. EBV latent infection is generally asymptomatic in immunocompetent individuals, but in immunocompromised persons it can produce EBV-driven immunoblastic sarcomas as well as an increased incidence of Burkitt's lymphoma (30). The EBV latent membrane protein LMP1 is the principal EBV oncoprotein and acts as a constitutively active, ligand-independent, receptor-like molecule. Signaling from LMP1 mimics many aspects of CD40 signaling (reviewed in reference 21), and recent studies indicate that LMP1 signaling incorporates components of TLR pathways as well (25, 33). LMP1 contains two domains in its C terminus, C-terminal activation region 1 (CTAR1) and CTAR2, that are predominantly responsible for its signaling capacity. CTAR1 contains a PXQXT domain that binds members of the TRAF family (TRAF-1, -2, -3, and -5). CTAR2 contains a YYD motif necessary for association with TRADD and RIP (14). TRAF6 also participates in LMP1 signaling most likely through indirect interactions with LMP1 (25, 33, 39).

RIP is a death domain-containing adaptor protein that associates with TNF receptors (TNFR) and is essential for TNF-induced activation of NF-κB (12, 17, 36). RIP also participates in TLR signaling (27, 38) and has been implicated in activation of IRF3 in response to double-stranded RNA (dsRNA) but not formally linked to IRF7 (2). In addition, RIP associates with LMP1 (14), although its role in LMP1 signaling remains obscure. RIP possesses serine/threonine kinase activity and undergoes autophosphorylation (12), although RIP's kinase activity is dispensable for many of its functions (13, 19, 36).

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▽ Published ahead of print on 12 February 2007.

RIP is modified by at least two forms of ubiquitination, K48-linked and K63-linked polyubiquitination (41). K48-linked ubiquitination typically leads to proteasomal degradation of proteins, whereas K63-linked ubiquitination is implicated in proteasome-independent functions that activate and/or regulate protein function (35). Ubiquitination of RIP is essential for its signaling functions (19), and K63-linked ubiquitin (Ub) chains have been proposed to provide a scaffold for the assembly of signaling complexes (15).

We have shown previously that LMP1 promotes the phosphorylation and nuclear accumulation of IRF7 (45). IRF7 is necessary for LMP1-induced up-regulation of Tap-2 expression (45) and has been implicated in LMP1-mediated priming of EBV latently infected cells for production of type I IFN (42). In this study, we examine LMP1 signaling events that lead to the activation of IRF7. We demonstrate that RIP is involved in activation of IRF7 by LMP1 and show that LMP1 promotes the ubiquitination of RIP as well as interaction of ubiquitinated RIP and IRF7. Moreover, LMP1 promotes RIP-dependent ubiquitination of IRF7 conjugated at least in part through Ub K63, suggesting that modification by Ub may modulate IRF7 activity.

MATERIALS AND METHODS

Cell lines. HEK293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS). $RIP^{+/+}$ $TNFR^{-/-}$ and $RIP^{-/-}$ $TNFR^{-/-}$ immortalized fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 2 mM glutamine. All other cell lines were maintained in RPMI with 10% FBS. SVT35 Jurkat T lymphoma cells (parental) and RIP-deficient 35.3.13 Jurkat cells have been described previously and were a gift from Brian Seed (Massachusetts General Hospital, Boston) (36). Raji is a Burkitt's lymphoma B-cell line. DG75-TA and DG75-LMP1 cell lines have been described elsewhere (9).

Plasmids and reagents. IRF7 in pcDNA3 and IRF7 in pcLMP1 have been described previously (46, 47). Flag-IRF7 in pCMV2 and the IFN-β promoter construct were gifts of John Hiscott (McGill University, Canada) and have been described previously (22). Wild-type RIP and kinase-dead RIP (kd-RIP) (K45A) were provided by William Cance (University of Florida, Gainesville). Hemagglutinin-tagged Ub (HA-Ub) and HA-K63-only Ub (see below) in pcDNA3 were generously provided by David Boone and Averil Ma (University of California, San Francisco). Dominant negative TRAF6 (TRAF6DN) was provided by Arnd Kieser (GSF National Research Center for Environment and Health, Germany). The IFN-stimulated response element (ISRE)–Luc construct and pRL-TK (where TK is thymidine kinase) were purchased from Clontech and Promega, respectively. IRF7 and RIP antibodies were purchased from Santa Cruz; LMP1 antibody (cs 1-4) was purchased from DAKO; and the Flag-M2, Ub, and HA antibodies were purchased from Sigma.

Transfections and luciferase assays. HEK293 cells in 12-well plates were transfected with 0.1 μg of IFN-β promoter reporter plasmid or ISRE-Luc construct, 0.1 μg of pcIRF7, and 0.1 μg of LMP1 or RIP unless otherwise noted. Empty vector was used to equalize the total amount of DNA in the transfections. pRL-TK (0.025 μg) served as an internal transfection control. SVT35 and RIP-deficient Jurkat cells (8.5×10^5) were transfected with 1.5 μg of IFN-β promoter reporter plasmid, 2 μg pcIRF7, 2 μg pcLMP1, and 1.5 μg of pRL-TK. All transfections were done with Fugene 6 transfection reagent (Roche). Luciferase assays were performed 18 to 24 h posttransfection with a dual luciferase assay kit (Promega).

Immunoprecipitations and immunoblot analysis. Raji, DG75-TA, and DG75-LMP1 cells were lysed in immunoprecipitation (IP) lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 5 mM dithiothreitol, 0.2 mM Na orthovanadate, and Complete protease inhibitors). Cell extracts (1 mg) were incubated with 2 μg IRF7 or RIP antibodies overnight at 4°C. Protein A/G-Sepharose beads (Santa Cruz) were added and samples incubated for 1 h at 4°C. Precipitates were washed five times with 10 volumes of IP lysis buffer and eluted by boiling the beads for 5 min in 2× sodium dodecyl sulfate (SDS) loading buffer. The eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes followed by immunoblotting with the indicated antibodies. HEK293 cells in 60-mm dishes and $RIP^{+/+}$ or $RIP^{-/-}$ immortalized fibroblasts in 100-mm dishes were transfected with combinations of Flag-IRF7, pcLMP1, and HA-Ub expression vectors with the use of Lipofectamine 2000 (Invitrogen). Cells were harvested 24 to 48 h posttransfection and lysed in IP lysis buffer. Cell extracts were incubated with 2 μg Flag M2 antibody overnight at 4°C. Protein A/G-Sepharose beads were then added, and samples were incubated for 1 h at 4°C and washed five times with 10 volumes IP lysis buffer. For sequential immunoprecipitations, the washed beads were resuspended in 50 μl 1% SDS and 1 mM dithiothreitol and heated to 95°C for 5 min. After removal of the beads by centrifugation, the eluted proteins were diluted 20-fold with IP lysis buffer and incubated with 2 μg IRF7 antibody overnight at 4°C. Protein A/G-Sepharose beads were added for 1 h at 4°C, followed by five washes with 10 volumes of IP lysis buffer. The immunoprecipitated proteins were eluted by boiling for 5 min in 2× SDS loading buffer and separated by SDS-PAGE followed by immunoblotting with the indicated antibodies.

RNA interference. HEK293 cells were transfected with RIP small interfering RNA (siRNA) oligomer, purchased from Santa Cruz, with the use of Lipofectamine 2000 according to the manufacturer's instructions. The specific sequence is not supplied by the manufacturer. The control siRNA, which is a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA, was also purchased from Santa Cruz. After 48 h, siRNA-treated cells were transfected again with 0.1 μg reporter plasmid, 0.1 μg pcIRF7, and 0.1 μg pcLMP1 with Lipofectamine 2000. Cells were harvested 24 h later. Luciferase assays were performed as described above.

Retroviral production and infection. pBabe/RIP1 was made by PCR, subcloning RIP1 into the pBabe/Hygro vector at the EcoRI/SalI site. 293T cells in 100-mm dishes were cotransfected with 7 μg pVSV-G and pGAG-pol and either 7 μg pBabe/GFP (where GFP is green fluorescent protein) or pBabe/RIP1 with the use of a CalPhos mammalian transfection kit (BD Biosciences, Bedford, MA). Supernatant fluids containing GFP or RIP1 retroviruses were collected 48 h later. For retroviral infections, $RIP^{-/-}$ cells were plated in 100-mm dishes and infected with GFP or RIP1 retroviruses in the presence of Polybrene (8 μg/ml). The cells were selected in hygromycin (Invitrogen) for 2 weeks (40).

RESULTS

RIP1 activates IRF7. Although we have demonstrated previously that LMP1 activates IRF7 and enhances its transcriptional activity (45), the mediators of this activation of IRF7 have not been identified. RIP interacts with LMP1 CTAR2 (14), but a specific role for RIP in LMP1 signaling has remained unassigned. However, RIP mediates activation of IRF3 in response to dsRNA (2), and thus we thought that RIP may mediate activation of IRF7 by LMP1. To test this hypothesis, we first examined whether expression of RIP enhances the transcriptional activity of IRF7. HEK293 cells, which do not express endogenous IRF7 under our culture conditions, were transfected with IRF7, RIP, and an IFN-β promoter reporter plasmid, a target of IRF7 and commonly used index of IRF7 activity. Cotransfection of IRF7 and increasing concentrations of RIP activated the IFN-β promoter in a dose-dependent manner. RIP's effect on this promoter was dependent on IRF7; very little if any activity was detected upon transfection of RIP alone (Fig. 1A). The results suggest that RIP activates IRF7.

We next used a kd-RIP (K45A) mutant to examine whether RIP's enzymatic activity is required for activation of IRF7. The combinations of IRF7 and wild-type RIP or kd-RIP both activated the IFN-β promoter (Fig. 1B). Thus, RIP's kinase activity is not required for the activation of IRF7.

RIP1 is involved in LMP1-mediated activation of IRF7. To test further whether RIP is implicated in activation of IRF7 by LMP1, we examined IRF7 transactivation of the IFN-β promoter reporter in response to LMP1 in RIP-deficient Jurkat cells. Whereas very little activity was detected upon transfection of IRF7 or LMP1 alone into parental Jurkat cells, pro-

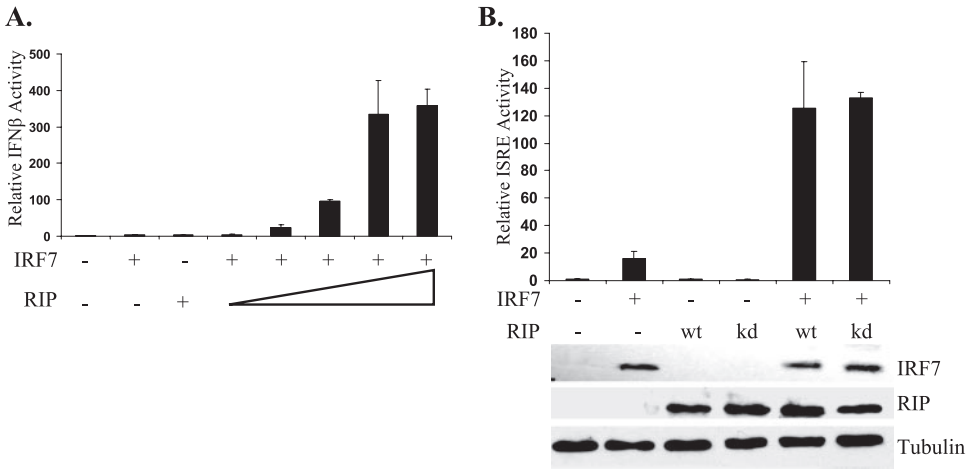

FIG. 1. RIP1 activates IRF7. (A) 293 cells were transfected with an IRF7 expression vector; 0, 10, 50, 100, 200, and 300 ng of RIP expression vector; and an IFN-β promoter luciferase construct. The 300 ng of RIP was used for the RIP-only sample. In all experiments, luciferase assays were performed 24 h after transfection to measure promoter activity. Results are the averages ± standard deviations of a representative experiment performed in triplicate. Similar results were obtained in three independent experiments. (B) 293 cells were transfected with combinations of IRF7 and RIP or a kd-RIP (K45A) mutant, which were expressed at similar levels as determined by Western blotting. wt, wild type.

moter activity was substantially increased upon cotransfection of IRF7 and LMP1, indicating that LMP1 activates IRF7 in these cells. However, the LMP1-plus-IRF7-dependent IFN-β promoter activity was reduced in RIP-deficient Jurkat cells compared with that in parental Jurkat cells (Fig. 2A). In addition, we used siRNA targeting RIP to suppress its expression in 293 cells (Fig. 2B) and then examined the ability of IRF7 to transactivate an ISRE-luciferase reporter, another common index of IRF7 activity, in response to LMP1. LMP1-plus-IRF7-dependent ISRE activity was reduced significantly after treatment with RIP siRNA oligomer compared with activity after treatment with control siRNA (Fig. 2C). Together these data demonstrate that RIP mediates activation of IRF7 by LMP1.

RIP1 and IRF7 interact. We next examined whether RIP and IRF7 interact. Raji is a Burkitt's lymphoma cell line that expresses RIP and due to latent EBV infection also expresses LMP1 and IRF7. Therefore, these cells are useful for examining the interaction of endogenously expressed RIP and IRF7. RIP and IRF7 were immunoprecipitated from Raji cell lysates with RIP and IRF7 antibodies, followed by immunoblotting for RIP. Two bands, neither detected with the control antibody, were observed upon immunoprecipitating RIP (Fig. 3A). The 74-kDa band corresponded to the expected size of RIP, while the other was a more slowly migrating, most likely modified, form of RIP. RIP is known to be ubiquitinated in response to TNF-α (6, 20, 49), and it is likely that RIP is ubiquitinated in this system as well. Therefore, to determine if ubiquitinated proteins immunoprecipitate with RIP, the samples were immunoblotted with an antibody to Ub, and bands, corresponding to the size observed for the modified form of RIP, were detected (Fig. 3B). These results indicate that RIP is ubiquitinated in Raji cells. Interestingly, ubiquitinated RIP specifically coimmunoprecipitated with IRF7 while very little if any unconjugated RIP was associated with IRF7 (Fig. 3A). Together, these data suggest that IRF7 preferentially interacts with ubiquitinated RIP in a physiological setting.

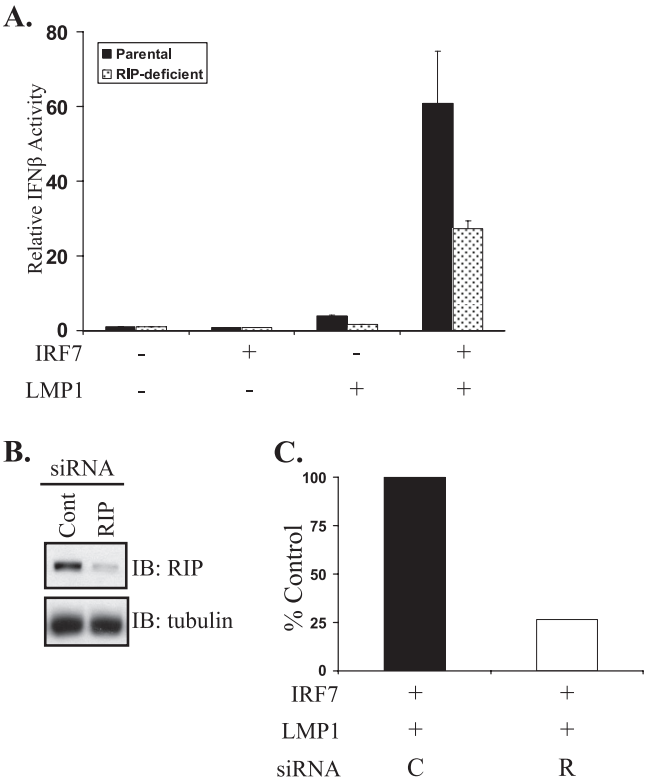

FIG. 2. RIP mediates LMP1-induced activation of IRF7. (A) Parental or RIP-deficient Jurkat cells were transfected with combinations of IRF7 and LMP1 with the use of Fugene 6. Results are the averages ± standard errors of triplicate samples. (B) 293 cells were transfected with control (Cont) or RIP siRNA (Santa Cruz) with the use of Lipofectamine 2000. RIP expression was analyzed by Western blotting 72 h posttransfection. (C) 293 cells transfected with control (C) or RIP (R) siRNA were incubated for 48 h before transfection with IRF7, LMP1, and an ISRE-luciferase reporter. Luciferase assays were done 24 h after the second transfection. Results were similar in three independent experiments. IB, immunoblotting.

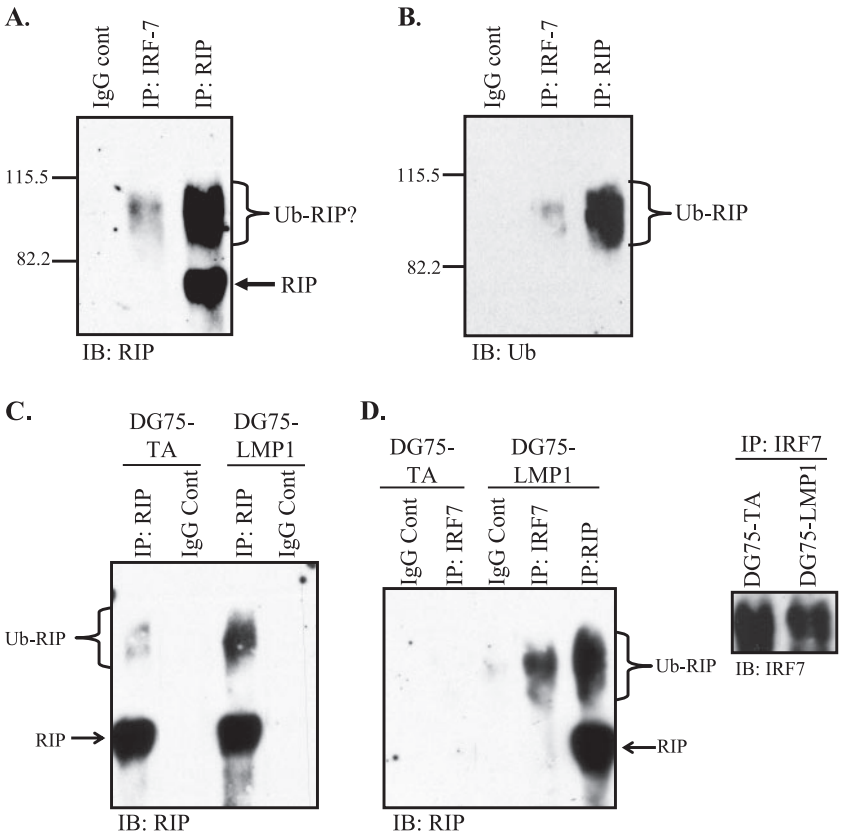

FIG. 3. LMP1 promotes ubiquitination of RIP and its interaction with IRF7. IRF7 and RIP were immunoprecipitated from Raji cell lysates with IRF7 and RIP antibodies, respectively. Normal rabbit immunoglobulin G (IgG) was used for control (cont) immunoprecipitations. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted (IB) with (A) RIP or (B) Ub antibodies. The arrow indicates RIP; modified forms of RIP are marked with braces. Molecular size markers (in kilodaltons) are noted at the left of blots. (C) RIP was immunoprecipitated from DG75 cells stably transfected with control vector (DG75-TA) or LMP1 (DG75-LMP1). Immunoprecipitated proteins were immunoblotted with RIP antibodies. (D) IRF7 was immunoprecipitated from DG75-TA and DG75-LMP1 cells. Immunoprecipitated proteins were immunoblotted with RIP antibody (left). RIP immunoprecipitated with RIP antibody is shown for comparison. Similar amounts of IRF7 were immunoprecipitated from both cell lines (right).

LMP1 stimulates ubiquitination of RIP. LMP1 signaling is constitutively active. The detection of substantial amounts of ubiquitinated RIP in Raji cells raises the question of whether LMP1 signaling promotes the ubiquitination of RIP. To examine the effect of LMP1 on RIP, we utilized DG75 cells stably transfected with LMP1 or control vector. DG75 is an EBV-negative Burkitt's lymphoma cell line that endogenously expresses IRF7 and RIP. RIP was immunoprecipitated from DG75-LMP1 or DG75-TA control cell lysates, and the amounts of unconjugated and ubiquitinated RIP were compared by Western blotting. Although levels of unconjugated RIP were similar in the immunoprecipitates from both cell lines, more ubiquitinated RIP was consistently detected in the LMP1-positive cells (Fig. 3C). These data indicate that LMP1 promotes ubiquitination of RIP and suggest that some signals transduced through RIP by LMP1 may be Ub dependent.

LMP1 stimulates the interaction of RIP with IRF7. The facts that RIP contributes to activation of IRF7 by LMP1 and that LMP1 promotes the ubiquitination of RIP and its association with IRF7 in cells suggest that interaction of RIP with IRF7 is part of the mechanism for activation of IRF7. Therefore, we next used DG75 cells stably transfected with LMP1 to ask specifically whether LMP1 promotes the association between RIP and IRF7. Immunoprecipitation of IRF7 from DG75-LMP1 or DG75-TA cell lysates followed by immunoblotting for RIP revealed that ubiquitinated RIP interacted with IRF7 in DG75-LMP1 cells (Fig. 3D, left), similarly to what was observed in Raji cells (Fig. 3A). RIP immunoprecipitated with RIP antibody from DG75-LMP1 cells was included to provide a size marker for unconjugated and ubiquitinated RIP. However, we detected little if any RIP coimmunoprecipitating with IRF7 from DG75-TA cells (Fig. 3D, left), although similar amounts of IRF7 were immunoprecipitated from both the LMP1-positive and the LMP1-negative cell lines (Fig. 3D, right). These data suggest that LMP1 stimulates the interaction between endogenous IRF7 and ubiquitinated RIP.

LMP1 stimulates ubiquitination of IRF7. The interaction between endogenous RIP and IRF7 in Raji cells was detected in both experimental sequences: immunoprecipitation of RIP followed by immunoblotting for IRF7 revealed that a small amount of IRF7 immunoprecipitated nonspecifically with the control antibody. However, much more IRF7 coimmunopre-

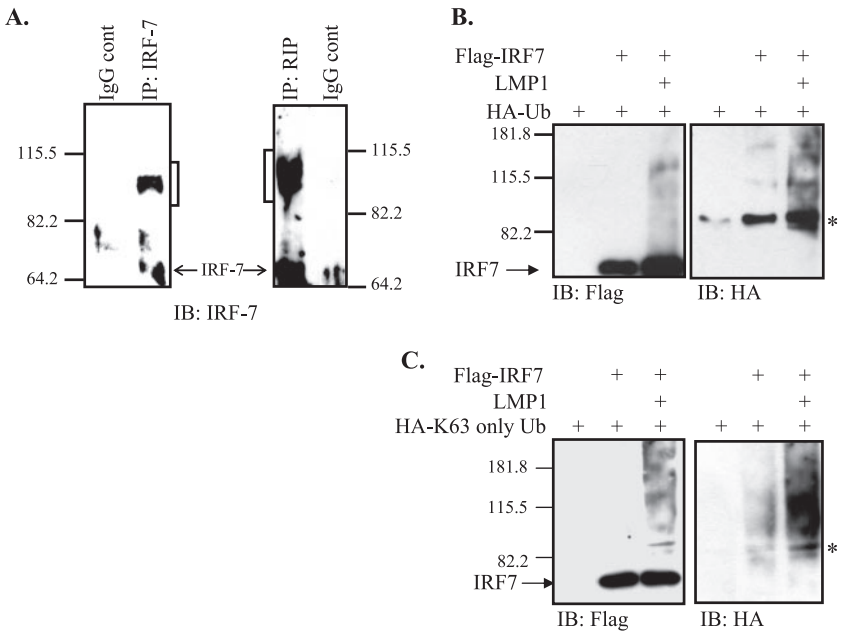

FIG. 4. LMP1 promotes ubiquitination of IRF7. (A) IRF7 coimmunoprecipitates with RIP from Raji cell lysates. Proteins immunoprecipitated with IRF7 or RIP antibodies were immunoblotted (IB) for IRF7. IRF7 is indicated by an arrow; high-molecular-weight, modified forms of IRF7 are indicated by brackets. IgG cont, immunoglobulin G control. (B) Combinations of Flag-IRF7, LMP1, and HA-Ub expression vectors were transiently transfected into 293 cells. Proteins were immunoprecipitated with Flag antibody and immunoblotted with Flag or HA antibodies. (C) Combinations of Flag-IRF7, LMP1, and HA-K63-only Ub vectors were transiently transfected into 293 cells. Proteins immunoprecipitated with Flag antibody were boiled and reprecipitated with IRF7 antibody prior to immunoblotting with Flag or HA antibodies. The asterisk indicates a nonspecific band. Molecular size markers (in kilodaltons) are noted next to blots.

cipitated with RIP (Fig. 4A). Interestingly, high-molecular-weight, most likely modified, forms of IRF7 were detected upon immunoprecipitation of IRF7, and these modified forms of IRF7 also interacted with RIP (Fig. 4A). IRF7 has recently been shown to be activated in a Ub-dependent manner (16), and the diffuse nature of the band is consistent with ubiquitination. Moreover, ubiquitinated proteins were immunoprecipitated with IRF7 from Raji cell lysates (Fig. 3B).

That modified—probably ubiquitinated—IRF7 interacts with RIP in Raji cells suggests that IRF7 may be ubiquitinated in response to LMP1 signaling. Therefore, we determined whether LMP1 promotes ubiquitination of IRF7. After transfection of 293 cells with HA-Ub and Flag-tagged IRF7 expression vectors alone or together with an LMP1 expression vector, cells were lysed and IRF7 was immunoprecipitated with Flag antibody followed by immunoblotting for HA or Flag. Cotransfection of LMP1 and IRF7 produced additional slowly migrating forms of IRF7 not readily detected in the absence of LMP1 (Fig. 4B, left). Furthermore, consistently more Ub was immunoprecipitated from lysates containing both IRF7 and LMP1 (Fig. 4B, right). Together these data suggest that LMP1 promotes ubiquitination of IRF7.

Several types of polyubiquitin chains linked through distinct lysines of Ub may have different functional outcomes. Polyubiquitination linked through K48 targets proteins for proteasomal-dependent degradation. However, polyubiquitination linked through other Ub lysines, such as K63, can have proteasome-independent functions, which include regulating protein-protein interactions and activation of signaling molecules, such as IKK (35). Therefore, we next asked whether LMP1 can promote K63-linked ubiquitination of IRF7. A Ub vector in which all lysine residues except K63 are mutated to arginine (HA-K63-only Ub) (4) along with Flag-IRF7 alone or in combination with LMP1 was transfected into 293 cells. Sequential immunoprecipitations were used to favor detection of ubiquitinated IRF7 but not ubiquitination of factors associating with IRF7. Proteins immunoprecipitated first with Flag antibody were heated to 95°C for 5 min in 1% SDS, followed by a second immunoprecipitation with IRF7 antibody. Interestingly, immunoblotting for HA revealed low levels of spontaneous ubiquitination of IRF7 upon cotransfection of K63-only Ub. However, addition of LMP1 clearly increased the amount of ubiquitinated IRF7 (Fig. 4C, right). Moreover, immunoblotting for Flag revealed a high-molecular-weight smear, consistent with an increase in ubiquitinated protein, in the LMP1-positive sample that was barely detectable in the LMP1-negative sample (Fig. 4C, left). These results indicate that LMP1 promotes K63-linked ubiquitination of IRF7.

Ub enhances IRF7 activity. That LMP1 promotes ubiquitination of IRF7 at least in part through K63-linked Ub suggests that Ub may regulate the activity of IRF7. To test further whether ubiquitination affects IRF7 transcriptional activity, 293 cells were transiently transfected with expression vectors for IRF7 and HA-tagged Ub together with an ISRE-luciferase reporter construct. The coexpression of IRF7 and Ub resulted in a dose-dependent increase in ISRE activity (Fig. 5A). Moreover, coexpression of IRF7 and the K63-only Ub mutant also increased ISRE activity (Fig. 5B). The results indicate that Ub enhances the activation of IRF7.

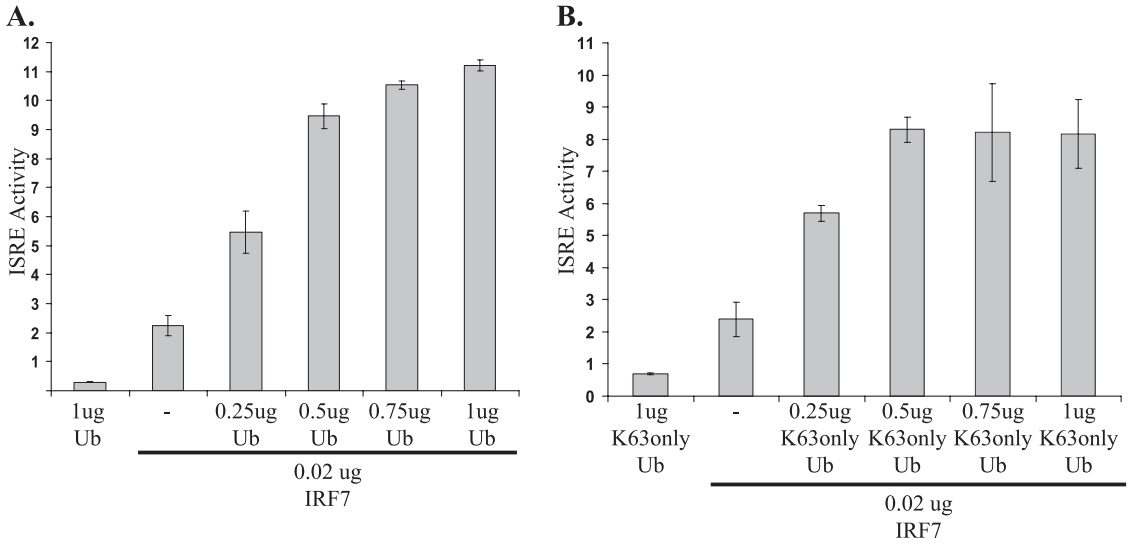

FIG. 5. Ub enhances IRF7 activity. 293 cells were transiently transfected with an ISRE-luciferase reporter and combinations of IRF7 and the indicated amounts of (A) HA-Ub vector or (B) HA-K63-only Ub vector. Results shown are averages ± standard deviations.

RIP contributes to LMP1-promoted ubiquitination of IRF7. Because RIP interacts with unconjugated and ubiquitinated IRF7 and contributes to activation of IRF7 by LMP1, we next asked whether RIP contributes to the ubiquitination of IRF7 promoted by LMP1. $RIP^{+/+}$ and $RIP^{-/-}$ immortalized fibroblasts were transfected with HA-Ub and Flag-IRF7 with or without LMP1. Flag-IRF7 was immunoprecipitated with Flag antibody. The samples were boiled and reprecipitated with IRF7 antibody before detection of ubiquitination by immunoblotting for HA. Ubiquitinated IRF7 detected in small amounts in $RIP^{+/+}$ cells in the absence of LMP1 was greatly increased by LMP1 (Fig. 6A, top). In striking contrast, ubiquitination of IRF7 was not detected in $RIP^{-/-}$ cells even in the presence of LMP1, although similar amounts of IRF7 were present in each sample (Fig. 6A, bottom). Finally, reconstitution of $RIP^{-/-}$ cells with RIP restored ubiquitination of IRF7 (Fig. 6B). The results indicate that RIP mediates LMP1-promoted ubiquitination of IRF7.

TRAF6 may be involved in LMP1-mediated activation of IRF7. TRAF6, a Ub ligase that catalyzes K63-linked Ub chains, interacts with and activates IRF7 in a Ub-dependent manner in response to TLR9 signaling (16). Therefore, we next examined whether TRAF6 is involved in LMP1-mediated activation of IRF7. After transfection of IRF7 and LMP1 or RIP with and without TRAF6DN into 293 cells, we assessed activation of an ISRE-luciferase reporter by IRF7. TRAF6DN blocked IRF7 activation by both LMP1 and RIP (Fig. 7), a result consistent with the idea that TRAF6 is involved in LMP1/RIP-mediated IRF7 activation.

DISCUSSION

IRF7 is the master regulator of IFN-α/β responses (11), and it also affects other target genes (28, 45). Since the stimuli are diverse, the mechanisms that govern the activity of IRF7 are likely to be complex and are important to understand. We have

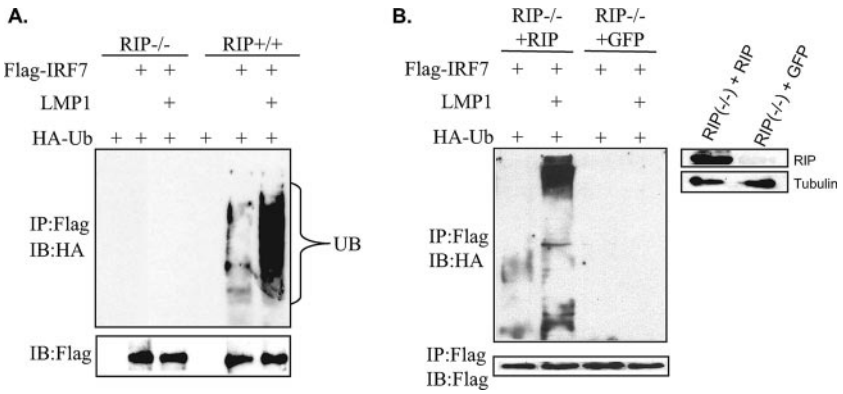

FIG. 6. RIP mediates LMP1-promoted ubiquitination of IRF7. Combinations of Flag-IRF7, LMP1, and HA-Ub vectors were transiently transfected into (A) $RIP^{-/-}$ or wild-type-RIP fibroblast cells or (B) $RIP^{-/-}$ fibroblasts reconstituted with RIP. Proteins immunoprecipitated with Flag antibody were boiled and reprecipitated with IRF7 antibody and immunoblotted (IB) with HA or Flag antibodies. RIP expression in reconstituted $RIP^{-/-}$ cells is shown on the right.

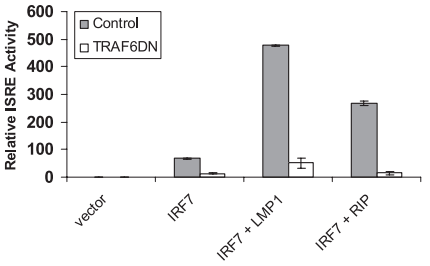

FIG. 7. TRAF6DN blocks IRF7 activation by LMP1 and RIP. Combinations of IRF7 and LMP1 or RIP with and without TRAF6DN were transfected into 293 cells. Activity of IRF7 was assessed with an ISRE-luciferase reporter. Results shown are averages ± standard deviations.

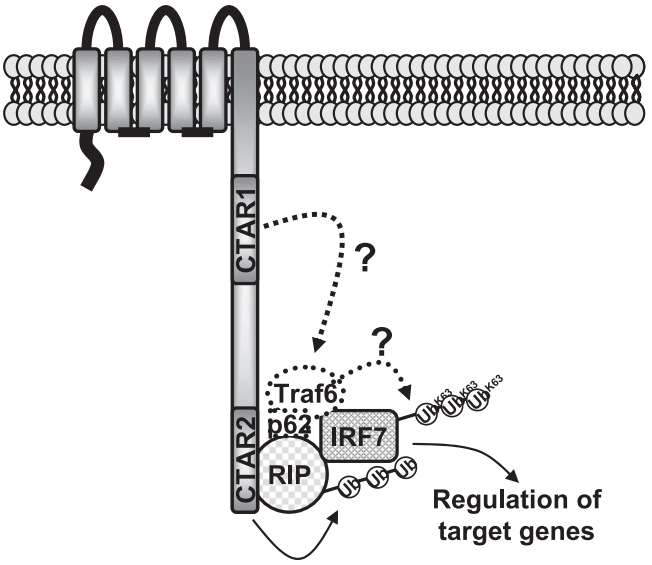

FIG. 8. Model for LMP1 activation of IRF7. LMP1 stimulates the ubiquitination of RIP associated with CTAR2. Ubiquitinated RIP functions as a scaffold where it interacts with IRF7 and possibly TRAF6 through p62. TRAF6 catalyzes K63-linked ubiquitination of IRF7 (indicated by a dotted arrow and a question mark), leading to enhanced IRF7 transcriptional activity. CTAR1-dependent activation of IRF7 may also involve TRAF6 (indicated by a dotted arrow and a question mark).

shown previously that LMP1 activates IRF7, promoting its phosphorylation and nuclear accumulation (45). Here we show that LMP1 promotes the ubiquitination of IRF7, which enhances its activity. RIP is a crucial element in mediating this ubiquitination, but activation of IRF7 does not require RIP's enzymatic activity. RIP has long been suspected to participate in LMP1 signaling, and we define here for the first time its important role. Finally, we show evidence that suggests that the regulatory ubiquitination of IRF7 brought about by LMP1 may use TRAF6 as an E3 ligase.

It may seem paradoxical that LMP1 upregulates (44) and activates IRF7, in light of its central role in the IFN response. However, IRF7's role may be in priming of latently EBV-infected cells by LMP1 for IFN production, the effect of which is to inhibit lytic EBV replication and superinfection by other viruses and thus to avert disruption of latency (42). Maintenance of latency is important for viral persistence because EBV, particularly the lytic antigens, generates robust cytotoxic-T-cell responses (30). In addition, LMP1 may utilize IRF7 for functions other than IFN responses. Indeed, LMP1 and IRF7 participate in a regulatory circuit that potentiates the expression of both factors (28). By increasing LMP1 expression, IRF7 may have an impact on LMP1's oncogenic potential (48).

The findings that IRF7 is modified by K63-linked Ub and the results of assays indicating that expression of Ub increases IRF7 activity are consistent with the idea that the activity of IRF7 in the LMP1 pathway is regulated in part by its ubiquitination. These findings certainly do not exclude the ubiquitination of IRF7 through other lysine linkages; in fact, IRF7 is known to undergo Ub-dependent degradation by the proteasome (43). In addition, Ub likely enhances IRF7 activity by promoting the activation of upstream kinases and signaling intermediates as well.

IRF7 is regulated in response to varied stimuli, and ubiquitination coupled in an undetermined sequence with phosphorylation may be a general mechanism for its activation. Both LMP1- and TLR9-induced activation of IRF7 invoke both phosphorylation and ubiquitination. Whether the two modifications are linked or can function independently is under study. The different degrees of IRF7 phosphorylation that follow viral infection may affect the magnitude of responses mediated by IRF7 (5). Ubiquitination of IRF7 would then provide an additional level of control that modulates these responses. Further, because polyubiquitin chains can facilitate the assembly of protein complexes (15), ubiquitination of IRF7 may modify its ability to interact with various transcriptional coregulators, thus influencing the selection of target genes.

LMP1 also promotes the ubiquitination of RIP and the interaction of ubiquitinated RIP with IRF7. Importantly in our study, preferential interaction of endogenous IRF7 with ubiquitinated RIP was detected under physiological conditions in EBV latently infected Raji cells. These findings probably have broader importance, as a complex containing RIP and IRF7 forms upon stimulation with poly(IC) as well (M. Kelliher, unpublished observations). Upon TNF stimulation, RIP is ubiquitinated (6, 20, 49), which is speculated to allow the recruitment of the TAK1/TAB2 complex (15) and is required for TNF signaling (19). Thus, our observations of ubiquitinated RIP specifically interacting with IRF7 may provide some insight into how LMP1 stimulates RIP to transduce signals.

Polyubiquitin chains are envisioned as functioning as scaffolds for the assembly of signaling complexes that bring kinases and substrates into close proximity (15). In the case of ubiquitinated RIP, in complex with IRF7, the signaling components might include a Ub ligase for IRF7, possibly TRAF6. TRAF6 is a likely candidate as an E3 ligase for IRF7 in LMP1/RIP signaling since it along with Ubc13 and Uev1A/Mms2 catalyzes K63-linked ubiquitination (7). Moreover, TRAF6 interacts with RIP through the adaptor protein p62 in IKK activation (31). TRAF6 also interacts with IRF7 (10, 16) and functions in the Ub-dependent activation of IRF7 in the TLR9/MyD88/TRAF6 pathway (16). Our data demonstrating that TRAF6DN blocks activation of IRF7 by both LMP1 and RIP are consistent with the idea that TRAF6 functions in the LMP1/RIP pathway as well. However, further studies with

several systems are needed to demonstrate whether TRAF6 is in fact responsible for the ubiquitination of IRF7 induced by LMP1. The RIP/IRF7 complex likely also contains an IRF7 kinase, such as TBK1 or IKKε. In fact, RIP, in conjunction with FADD and TBK1, is required for dsRNA-dependent activation of IRF3 (2). That RIP interacts with and promotes the ubiquitination and activation of IRF7 and that it is required for activation of IRF3 by dsRNA (2) suggest that RIP, in addition to its role in NF-κB activation, may be a general activator of these IRFs, facilitating the assembly of several potentially distinct signaling complexes.

IRF7 activity is reduced but not absent in RIP-deficient cells, and our data are compatible with additional mechanisms that are independent of RIP. RIP interacts with LMP1 CTAR2, and it is likely that RIP facilitates CTAR2-dependent activation of IRF7 (Fig. 8). LMP1 can also activate IRF7 via CTAR1 (45; data not shown). The signaling cascade downstream of CTAR1 for the activation of IRF7 is unknown and is likely independent of RIP but may involve TRAF6. TRAF6 colocalizes with LMP1 (33) and participates in signaling events downstream of both CTAR1 and CTAR2 (25, 33, 39). The role of TRAF6 in LMP1 signaling through CTAR2 and RIP but also independently through CTAR1 is likely to be defined as interrelated aspects of a complex mechanism and is under study.

ACKNOWLEDGMENTS

We thank all those who provided reagents as specified in Materials and Methods. We are grateful to Jenny Ting, Edward Gershburg, and Julia Shackelford for critical reading of the manuscript and thank Christopher Maier for technical assistance.

This work was supported by a grant from the NIH (R21-AI042371). L.E.H. was supported by an NCI training grant (T32-CA09156).

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